Characterizing bleaching response of *Marginopora vertebralis* for potential use as a bioindicator tool in reef assessment.

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Abstract

We propose to study the bleaching response of *Marginopora vertebralis*, a large benthic foram (LBF) species common to the reef systems around Tonga, a nation of islands in the South Pacific. LBFs share similar ecology to reef-building corals, and have been used as bioindicators to assess a variety of environmental variables. We propose that *M. vertebralis* is well suited to use as a bioindicator for the conditions that cause coral bleaching, and could greatly expedite the process of monitoring reefs for bleaching. To explore this potential, we aim to characterize the bleaching behavior that *M. vertebralis* undergoes in response to several environmental conditions known to be stressors for coral: increasing water temperature, visible and UV light irradiance, and high nitrate flux. By exposing specimens to varying intensities of these stressors under lab conditions, we will determine the thresholds and rate of bleaching response. Furthermore, we aim to develop and test the accuracy of a system for visually assessing bleaching in *M. vertebralis* that will facilitate future data collection and enable citizen science applications of this tool. An understanding of how bleaching in M. vertebralis corresponds to the environment, coupled with a system for visually assessing bleaching in the field, will allow the coloration of these forams to serve as a rapid and low-cost gauge of reef health.

Introduction

Symbioses with photoautotrophic microbes are a greatly beneficial asset in the sunlight-rich, nutrient poor tropical marine environments (Muscatine & Porter, 1977), and have arisen separately in a wide array of taxa, including sponges, cnidarians, flatworms, protists, and a few mollusks and chordates (McClanahan et al., 2018)

Such partnerships generally entail a host organism that houses photosynthesizing microbes (e.g. algae, diatoms, or cyanobacteria), either intracellularly or in specialized epicellular spaces (Kirk & Weis, 2016). The host receives the energy-rich compounds produced via photosynthesis in exchange for shelter and transferred essential nutrients such as nitrogen and inorganic carbon (Davies, 1984). In many cases, this relationship is obligate, with neither the host nor the symbiont species being able to survive in a symbiont-less (Aposymbiotic) state (Kirk & Weis, 2016). Other taxa appear to be facultative, containing symbionts have free-living life stages and can be cultured independently (Weis, 2008). Photo-symbioses are most common in organisms living in tropical waters, where the relatively low availability of nutrients is a limiting factor on the high energy demands of many animals (Muscatine & Porter, 1977).

Nowhere can the advantages of the photo-symbiotic lifestyle be more readily seen than in reef-building hermatypic corals of the order Scleractinia (Lough & van Oppen, 2018). The coral organism is a cnidarian polyp that buds into colonies of hundreds or thousands of genetically identical neighbors, cytoplasmically connected and supported through their shared aragonite

skeleton. These polyps harbors single-celled dinoflagellate algae of the genus *Symbiodinium* (called zooxanthellae) within their tissues (Lough & van Oppen, 2018). These symbionts provide the host with a steady flow of cheap energy and nutrients, providing an estimated 30% of the total nitrogen and 91% of the carbon needs of the host (McClanahan et al., 2018) and greatly facilitate the biomineralization process responsible for forming reefs (Lough & van Oppen, 2018). Reef-building corals pull calcium (Ca^{2+}) and carbonate (CO_3^{2-}) ions from seawater and crystalize these into aragonite skeletons (Stanley & Schootbrugge, 2018). This process is tightly coupled in coral to the photosynthetic activity of the symbionts, and calcification is, on average, three times higher in light than in darkness (Gattuso, Allemand, & Frankignoulle, 1999). Photosynthesis and calcification are often coupled in marine systems because the absorption of dissolved CO_2 by the algae creates a high-pH microclimate that favors calcium carbonate precipitation, a dynamic that is visible in even ancient forms of marine life such as stromatolites (Allwood et al, 2006). The exact mechanism by which photosynthesis impact calcification in corals is not yet understood (Stanley & Schootbrugge, 2018), but it widely agreed upon that the photosynthetic activity of corals is largely responsible for their ability construct reefs (Stanley & Lipps, 2011). In summary, the photo-symbiotic lifestyle of corals is responsible for the immense impact that corals have on their environments. The nutrient exchange allows them to prosper in an hostile and low-productivity environment, and the enhanced calcification allows corals to construct massive and complex reef environments that resist erosion and provide an immense web of ecological niches to exploit (Lough & van Oppen, 2018).

Bleaching

Although extremely advantageous when functioning properly, photosynthetic symbioses are vulnerable to environmental changes, and can sometimes collapse. In marine invertebrates, the breakdown of this partnership under stress and the ensuing loss of symbionts from the host is referred to as "Bleaching" (Lough & van Oppen, 2018). Although almost all marine organisms with photo-symbionts have been observed to bleach (Baird et al. 2009), this phenomenon particularly prominent in corals, including hydrocoral, scleractinian coral, and octocoral (McClanahan et al. 2018).

Although often perceived as an unnatural phenomenon, animals with photo-symbioses generally undergo seasonal fluxes in the density and pigmentation of zooxanthellae, tapering off at the end of the season with the highest average and maximum sea water temperatures (Douglas, 2003). These fluxes can occur within the organism even without visible color change (Fitt et al., 2000). However, an array of factors related to climate change have risen the threat of catastrophic, highly visible large-scale coral bleaching around the world, where severe and persistent loss of symbionts causes starvation and the collapse of reef ecosystems (Lough & van Oppen, 2018). These events are inexorably linked to human-caused climate change and have wide-reaching implications for the countless systems that depend on coral reefs (Hoegh-Guldberg,1999).

Bleaching behavior is a response to environmental stressors such as excess heat and light, viral and bacterial infection, ocean acidification, pollution, and salinity (Quigley et al., 2018). The underlying mechanisms are complex and varied, but evidence suggests that bleaching is frequently related to reactive oxygen species (ROS), generated by both the host and the

symbiont, that damage cell structures or act as virulence factors (Weis, 2008; Banin et al, 2003). ROS are generated by an overloading of photoprotective mechanisms in the photosystems and electron transport chains of the symbiont and host mitochondria, and hyperoxic environments created by excess photosynthesis (Oakley & Davey, 2018). Oxygen concentrations in corals reach up to 250% of saturation levels during the day in some cases (Kuhl et al, 1995). In normal cell function, ROS are detoxified and converted to H_2O_2 by the enzyme superoxide dismutase enzymes (SOD) within the symbiont and host (Oakley & Davey, 2018). If ROS production overwhelms ROS detoxification capacity, the leakage of ROS and ensuing cellular damage can cause an immune response by the host, leading to ejection or elimination of the symbiont via exocytosis, host cell detachment, or apoptosis (Weis, 2008). In other cases however, the role of ROS seems to be almost opposite— acting as a immunological defence system that is undermined by invading pathogens during bleaching conditions. For example, the mediterranean coral *Oculina Patagonica* bleaches as a result of bacterial infection by *Vibrio shiloi,* which produces SOD enzymes as a virulence factor that allow the pathogen to evade the coral's immune response by neutralizing antibacterial ROS released by phagocytes (Banin et al., 2003).

Another, related mechanism by which symbionts can produce excess oxygen and cause bleaching is through uncontrolled symbiont proliferation. Typically, the reproduction of symbionts in coral tissue is tightly constrained by limiting nutrients such as nitrogen and phosphorus, arresting cell division and driving the exchange of excess carbon with the host (Dubinsky and Berman-Frank, 2001) when human pollutants such as agricultural runoff cause an influx of these limiting nutrients, the symbionts can proliferate out of control, forcing the host to eject some or all of them to avoid oxidative stress (Dubinsky and Berman-Frank, 2001).

In the past two decades, bleaching has become a global phenomenon of great concern. Starting with a massive bleaching event in 1997-1998, reef scientists have become aware of the sensitivity of coral reef ecosystems to environmental change and the potential for massive damage caused by warming oceans and cyclic weather patterns such as El Niño(Lough & van Oppen, 2018). Since then, examples of regional scale bleaching events have abounded, with more global-scale events in 2010, and successively from 2014-2017 (Figure 1)(Lough & van Oppen, 2018: Eakin et al. 2017).

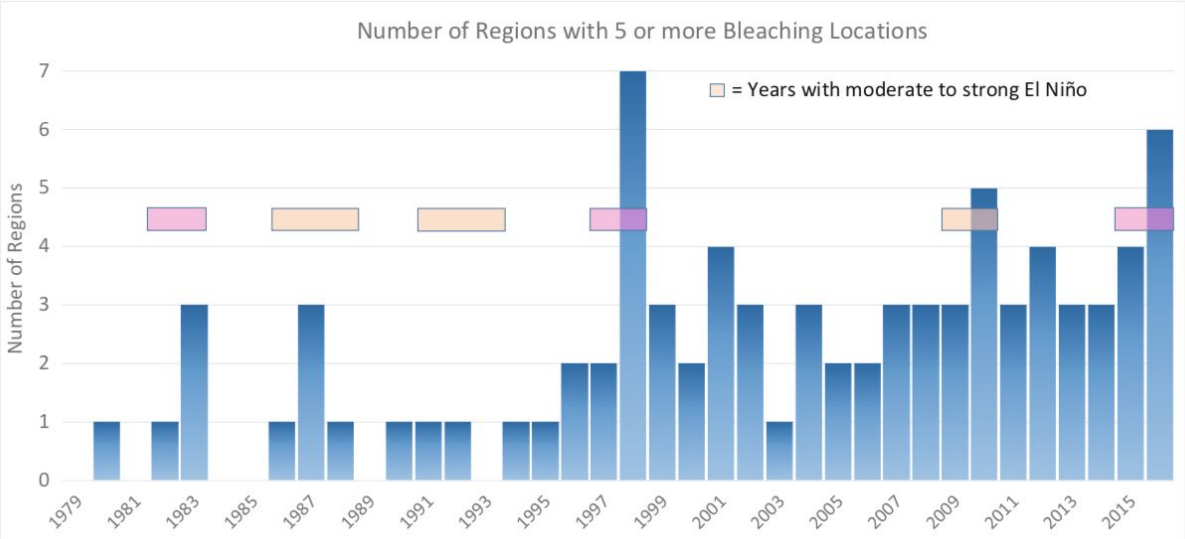
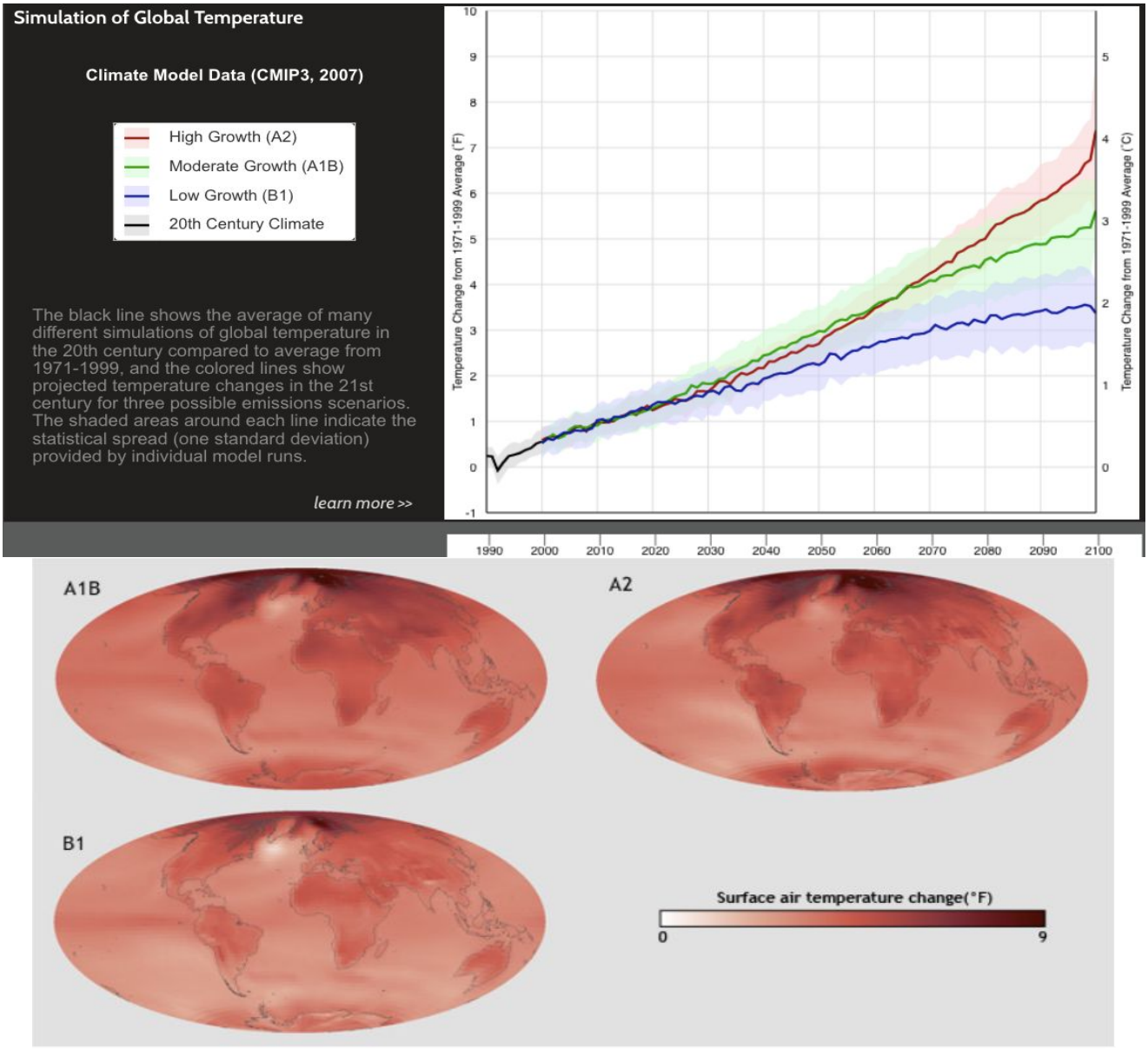


Figure 1. Number of regions reporting bleaching at a moderate to severe level in at least five locations per region. Coloured horizontal bars indicate years with moderate (orange) and major (pink) El Niño events. (Figure and caption from Oliver, Berkelmans, & Eakin, 2018)

The vulnerability of coral to changing environmental conditions is related to the relatively narrow range of conditions in which coral can live, constrained by light availability, bathymetry, temperature, and salinity (Lough, Heron, & Liu, 2018). Although coral systems have

evolved traits to recover from occasional ecosystem disturbances such as heat waves and tropical storms, this resilience requires periods of recovery (Buddemeier et al. 2004). Bleaching events present a threat to corals because loss of symbionts removes the corals' primary energy source, and can lead to starvation. Beyond that, the mechanisms of symbiont ejection, including host cell detachment and apoptosis, can often damage the host tissues and create risk of tissue necrosis or infection by pathogens (Oakley & Davy, 2018). Dead or dying coral skeletons can be also colonized by filamentous algae, increasing the difficulty of reseeding a reef after a die-off. Such a loss of coral abundance and biodiversity can undermine reef ecosystems and impact the ecological, economic, and cultural value of the reef.

The global climate is expected to warm by as much as 5 degrees on average by the end of the century as a result of human activities (Lough & van Oppen, 2018; IPCC, 2014)(Figure 2). This effect of rising air and sea surface temperatures is most pronounced at higher latitudes (Figure 2), but even relatively minor shifts predicted for the tropics pose a threat to reefs because many reefs have been found to live within 1-2 degrees of their bleaching temperature (Eakin et al., 2018).



These maps show the average of a set of climate model experiments projecting changes in surface temperature for the period 2050-2059, relative to the period from 1971-1999. The top left map corresponds with the green trend line above (IPCC scenario A1B); the top right map matches the red trend line above (IPCC scenario A2); and the bottom left map matches the blue trend line (IPCC scenario B1). All models project some warming for all regions, with land areas warming more than oceans. **large versions: A1B | A2 | B1** (Maps by Ned Gardiner, Hunter Allen, and Jay Hnilo, CICS-NC, using data courtesy the Coupled Model Intercomparison Project, or CMIP3.)

Figure 2. Projected magnitude and distribution of global temperature changes under different scenarios. Taken from https://www.climate.gov/news-features/understanding-climate/climate-change-global-temperature-projections (Herring, 2012)

The threat of increased temperature through climate change is the most prominent impact of human activity on coral, but myriad other human caused stressors can also contribute to weakening reef resilience, including include harmful fishing practices, nutrient and pollutant runoff, and coastal development (Ateweberhan et al., 2013; Lough & van Oppen, 2018). The regions that lack the resources to curtail these harmful practices and develop sustainable management practices are often those that are the most heavily dependent on coral reefs (Eakin et al., 2018). As the myriad impacts of human industrialization and climate change develop, bleaching events will only worsen in the future (Lough & van Oppen, 2018), and the threat of bleaching conditions is already forecasted to be severe in the coming months (Figure 3) Coral bleaching is a real and increasing threat to reef ecosystems and those that rely on them around the world.

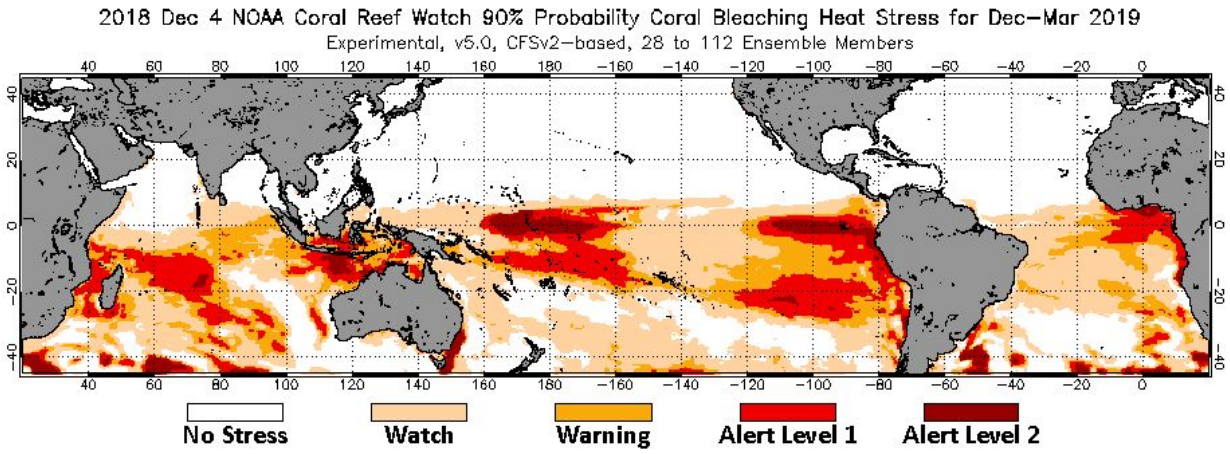


Figure 3. National Oceanic and Atmospheric Administration projection of bleaching threat for the beginning of 2019. Warning (orange) = possible bleaching, Level 1 (light red) = bleaching likely, Level 2 (dark red) = Mortality likely.

Dealing with bleaching

The monitoring of coral bleaching events and conditions has become a major focus of reef conservation efforts, and an critical part of understanding the phenomenon and its causes (Canten

& Spalding, 2018). Fine-scale local observation of reef health and corresponding environmental conditions not only help detect bleaching events as they occur, but contribute to a better understanding of the bleaching process and patterns of survival within or between communities (Canten & Spalding, 2018).

Current reef assessment techniques largely aim to quantify reef color to detect bleaching events while or after they occur. These techniques vary slightly between studies, and are generally limited by an inverse relationship between the amount of area assessed and the accuracy achieved (Canten & Spalding, 2018). Broad, rapid survey methods are generally semi-quantitative free swims or tows behind a boat, which can record low-level bleaching information over a large swath of land but are inadequate for numerical studies. Fixed transects, generally over a length of 100-500m, provide a better level of quantitative data, but often require snorkel or SCUBA experience and the capacity to record and review detailed video footage. Finally, Quadrats and line-point intercepts assess small, regularly interspersed areas in high detail, but at the cost of only covering a very small portion of the reef (Canten & Spalding, 2018). These measurements often need to be accompanied by environmental measurements, of Salinity, pH, temperature, and nutrient contents that can require advanced equipment, costly assay kits, and proper training and education to use (Eakin et al., 2018). Remote sensing of both bleaching and water temperature data via aircraft or satellite imagery are being employed to expedite these processes, but are still costly and relatively low resolution (Canten & Spalding, 2018).

In short, the pressing need for ongoing monitoring and assessment is at odds with the large cost of doing so effectively, which creates a dilemma for the residents of developing nations who rely

most on the health of their reefs. Furthermore, existing study methods focus on tracking bleaching events during or after the fact, generally creating meaningful data only after it is too late to intervene or address the problem. Especially in places where reef bleaching can represent an imminent threat to local economies and food supplies, the need for rapid, cost-effective, and actionable information on reef health is dire.

Bioindicators

The difficulties of assessing large, complex ecosystems with limited resources have presented a challenge to environmental scientists for decades (Soule & Kleppel, 1987). One promising tool to help expedite this task is the use of bio-indicators: sensitive species whose health or abundance can be used as a living metric of hard-to-measure environmental variables (Colon, Hallock, & Green, 2009). Living indicators of environmental conditions have been observed and interpreted by humans for millennia, from the somewhat mythical prairie-dog's shadow indicating the length of winter, to the very practical use of canaries to detect gas buildup in mines (Soule & Kleppel, 1987).

In ecosystems where many environmental conditions may interact to produce unpredictable impacts, bioindicators can be particularly useful because they automatically integrate these various conditions into meaningful information about their concrete impacts (Soule & Keppel, 1987). Bioindicators can also record the impacts of transient environmental stressors even if the conditions themselves change before they can detected by conventional methods (Jamil, 2001).

Bioindicators can come in many forms, and can be viewed at many levels of organization, from the level of physiological changes within individual organisms, to changes in population-scale abundance and health (Jamil, 2001). Foraminifera (forams) are one type of organism particularly suited to use as an indicator in aqueous environments. Forams are ideal bioindicators because of their small size, abundance, and sensitivity to environmental changes (Hallock, et al., 2003). They are dominant constituents of reef environments, contributing an average of 10-15% of the calcareous sediments around reefs, and make up as much as 60% of the sediment with their calcite shells (called "tests") in some basins around the reef (Ross, 1972). Specifically, large benthic forams (LBFs) have several attributes that make them uniquely applicable to sensing environmental conditions in reef environments. LBFs are characterized by a lifestyle similar to that of coral, using a combination of heterotrophy and photosynthetic endosymbionts to prosper on the seafloor of sunlight-rich waters and grow to sizes many times larger than other foram taxa (Hallock, 1985).

Among modern species, these traits are particularly embodied by *Marginopora vertebralis,* an LBF common to the Great Barrier Reef, Hawaii, Japan, and islands and atolls throughout the South Pacific, (Ross, 1972; Gudmundsson, 1994). *M. vertebralis* shares much of its habitat and lifestyle with reef-building coral in the tropics, living on the shallow seafloor and hosting an array of dinoflagellate algae taxa as symbionts, including members of 4 clades of *Symbiodinum* (Momigliano & Uthicke, 2013). These symbionts give their disc-shaped calcite tests a yellow-brownish green color that is easily visible to the naked eye due to their unusual size, commonly larger than 12mm in diameter and sometimes up to 3 cm (Ross, 1972).

The reefs around the islands of Tonga harbor unusually large and widespread populations of *M. vertabralis*. Unpublished work by myself and Dr. Sharyn Golstein of the University of Canterbury has positively identified and characterized samples from this Tongan population, and recorded observations about their ecology in the area. The living and dead tests of *M. vertebralis* are visible on the sand or encrusting other biota in or around several reef environments near the islands of Tongatapu and ‘Atata, and their tests alone comprise an estimated 60% of the soil in shallow seagrass environments bordering the reefs. Although research on other localities in the pacific have noted high concentrations of up to 100 large individuals per square meter (Ross, 1972), abundance at this level appears to be an anomaly of the Tongan population. In general, the Tongan population has been poorly researched compared to other localities such as the Great Barrier Reef (e.g. Ross, 1972; Reymond, Uthicke, Pandolfi, 2011; Reymond et al., 2013; Schmidt, Kucera, & Uthicke, 2014).

A bioindicator for bleaching conditions could be an immensely valuable tool. Although reefs themselves represent a kind of bioindicator of harmful environmental conditions, even early symptoms of coral bleaching often come too late for any action to be taken to help reduce stress to the reef, and color changes associated with low-level are often invisible to the naked eye (Fitt et al, 2000). The need to assess coral coloration *in situ* presents another difficulty because of the color-distorting effects of water, furthering the utility of a bioindicator that could collected and assessed on land or on a boat. If a more sensitive, more easily-surveyed organism could help forecast the onset of bleaching events before they impact the reef, it may also allow time for action to reduce the severity of the bleaching event. Many contemporary strategies for managing bleaching events include the reduction of secondary stressors, on the principle that doing so may

reduce the overall synergistic effect of stress on the organism (Baker et al., 2008). With early warning about bleaching risk, reef managers might respond by taking local action to protect against further damage from dredging, overfishing, and local pollution or development (Berkelmans, 2018). However, the idea that corals become more resilient to bleaching in the absence of secondary stressors is still a matter of some debate (Baker et al., 2008). While some fisheries management guides recommend such measures (e.g. Marshall & Schuttenberg, 2006; McCook et al., 2007) and others cite reefs where secondary damage after bleaching prevented recovery (Ateweberhan et al., 2013; Graham et al., 2008), and others argue that secondary stressors help weed out disturbance-sensitive individuals (Côté and Darling, 2010) or point to examples where bleached reefs have recovered more slowly within marine protected areas (McClanahan et al., 2001; Graham et al., 2008).

Beyond stress mitigation, much recent work has focused on other methods for supporting reef resilience, including engineering reefs to be more resistant to bleaching via human-assisted evolution of zooxanthellae (Chakravarti et al, 2018), CRISPR engineering of genes associated with bleaching (Cleves et al., 2018), and "re-seeding" reefs with introduced coral larvae (Cruz and Harrison, 2017). Early and improved detection of bleaching via a bioindicator could help manages apply such innovative approaches before and after bleaching events. Regardless of the specific approach taken, few would argue that advance knowledge of bleaching events would not be useful to fisheries managers, and fewer still would argue that methods to expedite reef monitoring are not needed.

Benthic forams have much potential for use as bioindicators; their small size, abundance, ease of collection, and sensitivity to environmental conditions are all valuable traits for collecting

environmental data (Hallock, et al., 2003). Several tools for using forams and foram communities as bioindicators have already been developed. Existing approaches generally use the proportion of certain taxa within the overall foram community as an indicator of environmental conditions; Alve (1995) noted a gradient of increasing foram biodiversity away from pollution sources in estuaries that could be used to track water quality, Hallock et al. (2003) established the FORAM index for assessing marine nutrient levels based on the proportion of small opportunistic taxa to large photo-symbiotic taxa (including *M. vertebralis),* and Colon, Hallock, & Green (2009) established that specific pollutants have predictable effects on foram test morphology. Surprisingly however, little work has been done to explore the possibility of bioindicators for reef health. Benthic forams have been shown to eject symbionts in response to several of the same stressors that cause coral bleaching, Hallock & Talge (1993) were among the first to document foram bleaching in the diatom-bearing species *Amphistegina gibbosa* in the Florida Keys associated with irradiance, and Richardson (2006) documented bleaching of the soritid dinoflagellate-bearing species *Sorites dominicensis* in Florida and Belize associated with high water temperatures and irradiance. Furthermore, test color in forams has been shown to correlate with endosymbiont activity in numerous diatom-bearing taxa (Hallock et al., 2006; Uthicke et al., 2012) and the dinoflagellate-bearing species *Baculogypsina sphaerulata* (Hosono, Fujita, & Kayanne, 2012). Hallock et al. (2006) even proposed the use of *Amphistegina* bleaching in as an indicator of reef stress conditions, but noted that this genus dominantly bleaches in response to irradiance, rather than heat stress. Adult *Amphistegina* tests are also smaller than 1mm,, and would require laboratory assessment to observe bleaching. A more powerful tool would use a highly abundant *symbiodinium*-hosting species that is large and

colorful enough to be assessed in the field (Figure 4), which would allow for rapid and resource-efficient data collection and a greater relevance to *symbiodinium* in coral. *M. vertebralis* has great potential for this role.

Figure 4. Samples of Marginopora vertebralis collected in Tonga, showcasing the large and colorful tests of adult specimens

Having access to such a bioindicator tool could revolutionize the formidable task of reef assessment in resource-poor areas like Tonga, where the country's reef resources are distributed across 700,000 km^2 of marine territory and some 50 distinct reef systems (Lovell & Palaki, 2002). An easy-to-use bioindicator tool could allow anyone with minimal training to collect

meaningful data about ecosystem health, and potentially allow proactive measures to protect reefs from worsening conditions. Furthermore, the low level of education and training needed for using such a tool could allow the implementation of citizen science approaches to data collection, and support a greater effort to unite the civilians population of Tonga with fisheries managers in a collective effort to protect of their marine resources. Because *M. vertebralis* has been catalogued throughout Micronesia, Melanesia, Polynesia, the GBR, and Japan (Gudmundsson, 1994), any bioindicator tool developed in Tonga may also have wide-reaching applications throughout the pacific.

Although the potential for a foram-based bioindicator of bleaching conditions is clear from past work with forams and the unique physical characteristics of *M. vertebralis*, much work still needs to be done to elucidate the relationship between bleaching behavior in forams and that of coral. Specifically, this research will focus on determining the causes, sensitivity and timing of bleaching in *M. vertebralis*, and how predictable or quantifiable the resulting color shift is. This understanding can be combined with field work assessing the correlation between foram bleaching and the health of nearby reefs to determine if and how the symbiont activity of *M. vertebralis* can be used as a predictor of bleaching events.

Methods

Field work

Field observations will be necessary to determine what relationship, if any, exists between *M. vertebralis* health and the health of neighboring coral reefs in Tonga. Reef health will be

assessed using the Coral Health Index (see Kauffman et al., 2011), which is calculated in proportion to the abundance and diversity of fish present, the percentage of the seafloor area covered by live coral and crustose crystalline algae, and the inverse proportion of the abundance of *Vibrio* pathogenic bacteria. If any correlation is evident to suggest that the symbiotic health of these organisms is linked, two study areas with healthy and dense *M. vertebralis* populations will be established in Tonga, in the areas studied extensively by students of Frontiers Abroad research trips to Tonga (Figure 5). One of these study sites (Figure 5 B, b) is in the shallow seagrass environment near the Sopu shelf reef bordering the island of Tongatapu, near the Tongan capital of Nuku'alofa. The other site (Figure 5 A, a) is a seagrass environment inshore of a barrier reef by the smaller, less populous island of 'Atata.

These sites are ideal because they represent a diversity of reef conditions, including reef type, proximity to urban pollution sources, and heaviness of human traffic. These sites also have the benefit of having several years of detailed environmental data recorded by Dr. Sharyn Goldstein and her undergraduate students during field camp work in this area.

Figure 5. Study sites in Tonga for the collection and observation of *M. vertebralis* populations. Box A shows the area around the small, lightly populated island of ‘Atata, with the specific study site shown in box “a” (coordinates 21°03’11”S 175°15’25”W). Box B shows the area on the larger, urbanized island of Tongatapu, with the specific study site shown in box b (21°07’35”S 175°12’22”W).

In each of the study locations, foram samples will be collected according to the recommendations of Schönfeld et al., (2012), by using a rigid scoop and 50cm^2 container to collect foram samples from between 0 and 1 cm below the sediment surface. Five 50 cm^2 samples will be collected using judgement sampling, selecting for areas within the study site containing abundant large specimens. Judgement sampling is appropriate because this data collection does not aim to determine the variability within the overall population, but rather the mature sub-populations most useful for potential as a bioindicator. The collected samples will be washed on a 2mm^2 screen using seawater to separate large *M. vertebralis* specimens from sediment and other forams. The separated *M. vertebralis* specimens will then be categorized by size and color to determine the natural color and size variation within and between populations of *M. vertebralis*; Large foram tests (>1 cm diameter, as defined by Ross (1972)) will be separated from the sample and counted to determine the density of large specimens in the area, and qualitative observation of the natural coloration spectrum that exists in all study areas will be used to create a preliminary 6-step color-scoring standard similar to those developed by The University of Queensland for citizen science data collection of coral bleaching (Figure 6)(CoralWatch, 2018). This reference will allow for collection of quantitative data via visual assessment during the remainder of the experiment.

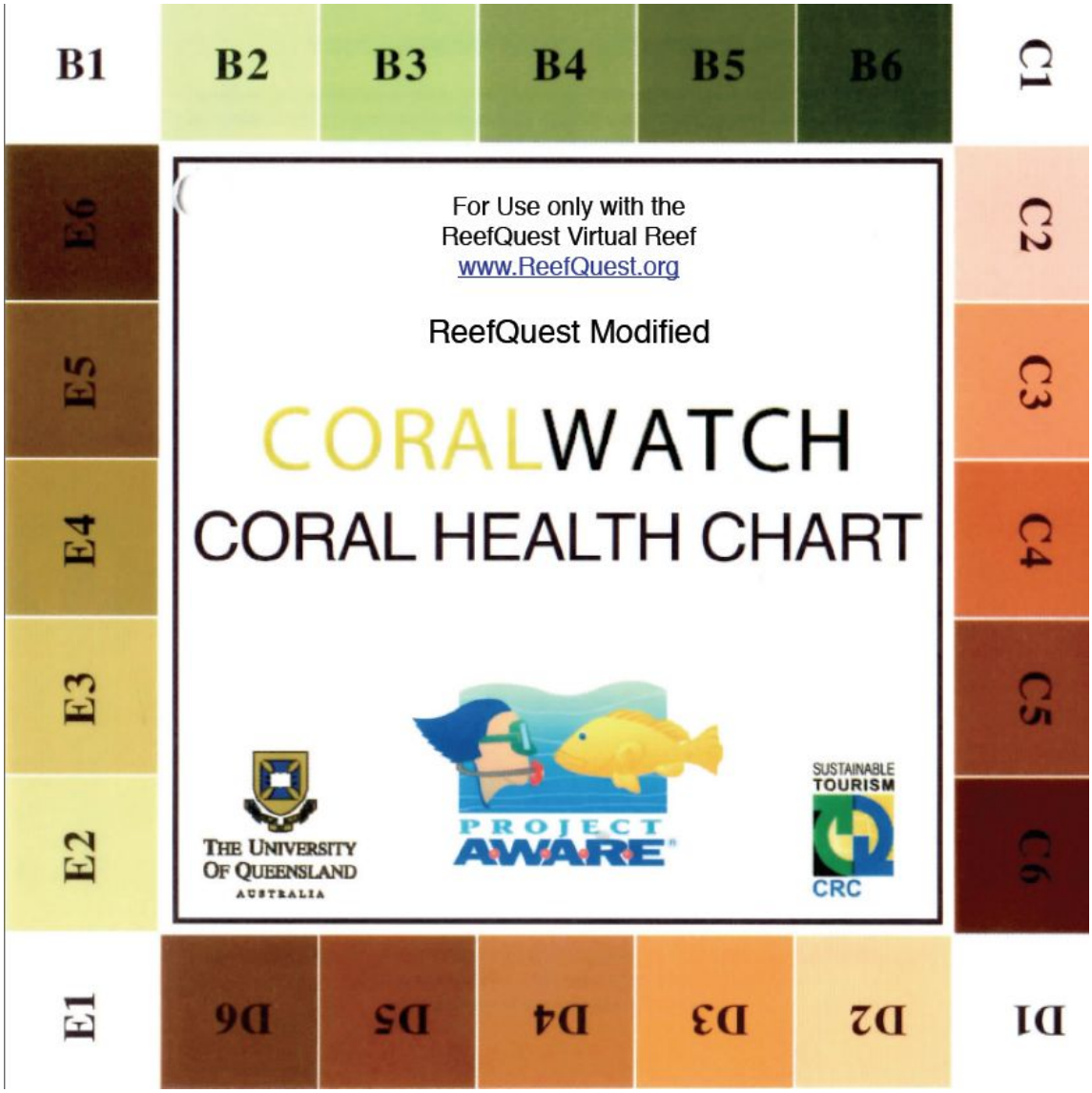


Figure 6. A model for the visual color assessment tool we aim to create for *M. vertebralis* after determining what range of test coloration exists in natural populations. From CoralWatch (2018).

After assessing color range and density of large individuals, the specimens separated from each sample will be vital stained according to the recommendations of Schönfeld et al. (2012), by immersing them in a beaker with 2 gL^{-1} Rose Bengal stain in seawater for 14 days. The results of this stain will indicate the proportion of live forams in each location, and can be

used to identify diagnostic visual signs to distinguish living organisms from dead ones in the field. A second round of sediment sampling and isolating large (>1 cm diameter) *M.vertebralis* specimens will then be conducted in the same manner as before within each study area. Based on the density of *M. vertebralis* observed at these locations (Snyder & Goldstein, unpublished data) We estimate that this will yield 400-700 individuals. These specimens will be transferred to 500ml glass containers with seawater for transport back to the laboratory, and will be used as experimental subjects during for the remaining lab work. Once in the lab, foram specimens will be cultivated using methods from Schmidt et al., (2011); housing 20 individuals per 500ml beaker indoors, maintaining temperature at 25°C and at light intensities similar to those at measures at the site of collection (expected to be approx. 45–50 umol photons m^{-2} s^{-1}, or 3,300-3700 lux of white light) using a Catalina Aquarium 96 Watt 10k/450Nm 50/50 Blue/White Compact light (Lakeport, CA) in a 12 hour light/dark cycle. Seawater will be replaced with fresh seawater (unfiltered, but with sediment settled out) every 3 days. This setup will minimize the need for repeat collection trips, as specimens maintained in this fashion have been found to be cytologically indistinguishable from newly collected specimens (Talge & Hallock, 2003).

Lab work

The overarching goal of this experimentation will be to assess specific bleaching parameters of *M. vertebralis* in order to compare them to known thresholds for coral bleaching. Alongside that, we aim to develop and test techniques for gaining meaningful, accurate information from visual assessment of the foram tests. Establishing a visual color-scoring system

will allow what is learned about the bleaching behavior of *M. vertebralis* to be applied towards the creation of a visual bioindicator of ocean conditions.

In the most ideal case scenario, the results for these experiments will show that *M. vertebralis* bleaches consistently, visibly, and sensitively in response to the stressors that cause coral bleaching. We further hope to find strong evidence that visual scoring of test color can be closely matched to quantitative measurements of symbiont activity.

Correlating test coloration with symbiont activity

First we aim to determine a rigorous quantitative correlation between foram test color and symbiont activity. Matching a specific level of symbiont density or photosynthetic activity to a corresponding test color will allow visual color-scoring to be used as a proxy for other, more work-intensive methods in the future.

20 specimens from each level of the visual color-scoring scale will be assessed using the methods of Sinutok et al (2013) for measuring photosymbiont activity by spectrophotometric assay for chlorophyll a and c_2 levels. After color scoring, the wet weight of each test will be recorded, then specimens will be homogenized using a glass rod in 90% 3 mL of acetone, and stored away from light at 4°C for 24 hours to extract chlorophyll. The extract will be centrifuged at 1500 g for 10 minutes, then the supernatant placed in a quartz cuvette and to test absorbance and 630 and 664nm with a Varian Cary 4000 spectrophotometer (Palo Alto, California) (Sinutok et al., 2013).

These data can be used to constrain the chlorophyll levels (and therefore symbiont activity) within foram tests at each level of the visual color-scoring system. Plotting absorbance

readings from each color-score category will show what range of symbiont densities are represented within each score. The r^2 value of the correlation will be considered the measure of how closely color score represents symbiont activity. Establishing a rigorous correlation between the semi-quantitative color-scoring system and an accepted quantitative measure of symbiont activity will allow color-score data to be used as a proxy for more work-intensive measurement methods.

As another measure of symbiont abundance, we will also collect data for individuals from all color categories on algal cell density by crushing the foram test with a glass rod in 3ml of filtered seawater and counting the number of symbiont cells per mm^3 using a LW Scientific CTL-HEMM-GLDR hemocytometer (Lawrenceville, Georgia)(Sinutok et al., 2013). Hemocytometer counts can be converted to density of algal cells per gram of foram test as a measure of the symbiont activity for that individual. These data will be plotted against visual color score in the same manner as the absorbance data in order to create another quantitative metric of symbiont activity that can be assessed visually.

Developing visual assessment criteria for viability

It will also be necessary to determine a reliable way of distinguishing live from dead forams visually. The literature is unclear as to the threshold of test breakage or deformation causes death of the foram, and how well the tests are preserved after the organism's death. To ensure that bioindicator data is collected only using live forams, reliable markers of viability need to be established.

Using the samples collected and stained with rose bengal during field work, individuals will be scored on a scale of 1-5 under a microscope for their presence of vital staining, degree of test integrity, and presence of epibiota (which has been noted as a characteristic of dead forams; Ross, 1972). In this way, characteristics that consistently indicate the death or near-death of the organism can be identified to allow future such forams to be excluded from data collection.

The efficacy of rose bengal staining will be confirmed with a static stop-flow respirometry test according to svendsen et al. (2016). After a pilot study to determine the appropriate amount of time needed to measure metabolic gas production effectively, stained (presumed to be alive) specimens will be placed in a sealed 20ml syringe chamber of filtered seawater to measure photosynthetic O_2 production under control light conditions. After the established time, the chamber will be flushed through a dual gas (O_2 / CO_2) respirometer (Columbus Instruments Oxymax ER, Columbus Ohio). The same process will be repeated, but shielded from light, to determine metabolic O_2 consumption. Total photosynthetic production of O_2 can then be calculated by subtracting the amount of oxygen consumed in the dark from the amount produced in the light. Presence of both metabolic and photosynthetic activity will confirm viability of the organism.

Assessing bleaching reactions of *M. vertebralis*

Once reliable visual cues of life and symbiont activity have been established, the exact environmental conditions that cause bleaching in *M. vertebralis* can be explored. Dinoflagellate-hosting Forams have been observed in the field to bleach under many of the same conditions as corals (Richardson, 2006), and specifically under increased heat (Schmidt et al.,

2011), light (Sinutok et al., 2013), and nutrient stress (Dubinsky, Berman-Frank, 2011). To determine exact thresholds for *M. vertebralis* bleaching in all of these conditions, populations of forams cultivated in the manner described above will be exposed to a single stress treatment at a range of intensities as described below. To keep track of foram coloration over time, each specimen in the experiments will be assessed with the visual color scoring method before, during, and after exposure to stress conditions. Bleaching for an individual will be considered as a decrease by at least one color-score level from its original color score. Alongside visual color scoring, we will periodically sub-sample each treatment group and perform spectrophotometric chlorophyll assays as described above, which will create a parallel data set with which to corroborate the visual color-scoring data and continually improve the rigor of the visual method.

Depending on the relationship that is determined between color score and absorbance (i.e. whether or not they correlate linearly), the absorbance data from sub-sampling may require a different definition of bleaching. If visual color score and absorbance can be linearly converted as hoped, then bleaching for the spectrophotometric data will be defined as the decrease in absorbance equivalent to one color score level. If this is not the case, bleaching will be defined as the first point at which absorbance decreases absorbance significantly from the original measurement.

Heat stress

Exposure to different temperature stress levels will be carried out based on the methods of Schmidt et al., (2011); 4 beakers of 20 forams each will be placed in each of five internally lit plant incubators (Thomas Scientific LI15 Diurnal Plant Growth Chamber, Swedesboro, New

Jersey) set at 23°C, 28°C, 30°C, and 32°C and maintained at the same light regime (approx 50 umol photons m^{-2} s^{-1}, 12h light/dark cycle) for a total duration of 30 days. To avoid thermal shock, the forams will be introduced into the test temperatures at the rate of 1°C per hour until achieving the desired water temperature. Color scoring of each specimen will take place once per day. Once every two days, 3 randomly-chosen tests will be sub-sampled, color-scored, and assayed for chlorophyll as previously described. At the end of the experiment, all remaining tests will be given a final color score and assessed via spectrophotometry as well. Bleaching data will be assessed using one-way ANOVA tests to determine whether test coloration (as measured by absorbance or mean color score) varied significantly from the beginning to the end of the treatment. Tukey's honestly significant difference post hoc test will be used to identify which time intervals yielded significant differences in coloration in order to determine how bleaching progressed over time. All tests will be performed with a 95% significance level. The timing of bleaching onset as determined by post-hoc analysis will help determine the speed and sensitivity of response to a given stressor. Comparing the ANOVA measure of overall color change between different intensities of the same stressor will help show the intensity threshold for bleaching in *M. vertebralis.*

Light intensity

Light stress from both photosynthetically active radiation (PAR) from 400–700 nm and from UV radiation (280– 320 nm) has been shown to harm both corals and benthic forams (Schmidt 2013). Here, the impacts of photo stress will be tested by modifying the control light conditions (50 umol photons m^{-2} s^{-1} / 3700 lux of white light in a 12 hour light/dark cycle).

Photosynthetically active radiation (PAR) will be delivered under 4 light conditions in the manner adapted from Williams and Hallock (2004), using the same lighting apparatus used for general laboratory cultivation to provide PAR, alongside a 20W UVB source (Phillips TL 20W/12 RS SLV/25, Hamilton, UK) will be used for 4 hours in the middle of the 12 hour light cycle to deliver UVB radiation to mimic the period of highest UV intensity around noon. For each treatment, 3 beakers of 20 forams each will be exposed to a constant light intensity 30, 60, 90, 120 or 150 umol m^{-2} s^{-1} in increasing increments of 30 umol m^{-2} s^{-1}, intended to represent a range from lower-than-natural irradiance up to highly stressful irradiance based on the control and stress-level light intensities for coral recorded by Oakley & Davey (2018)

All treatments will be shielded from UVC radiation (250–280 nm) by cellulose acetate film. Light levels will be modified according to Schmidt et al (2013) by using an adjustable rack to raise or lower the light source, and applying shade cloth over the beakers containing specimens as needed. Spectral irradiance levels will be checked with a UV-visible spectroradiometer (Spectral Evolution UDS-1100, Lawrence, Massachusetts) once daily, each time the color of the specimens is recorded. Specimens will be exposed to one experimental condition for a period of 30 days, during which they will be visually color-scored daily and 3 individuals sub-sampled every other day for chlorophyll assay, then color-scored and assayed at the end of the experiment. Data will be processed as before, using one-way ANOVA tests to assess whether test coloration (as measured by absorbance or mean color score) varied significantly from the beginning to the end of the treatment, and using Tukey's honestly significant difference post-hoc test to identify which time intervals yielded significant differences in coloration.

Nutrient levels

Nitrogen is one of the primary limiting nutrients that can cause excessive symbiont growth in coral, and ostensibly forams (Dubinsky & Berman-Frank, 2011). To test whether excess nitrogen availability causes uncontrolled proliferation of symbionts and bleaching in coral, we aim to test three nutrient levels (0.5 (control), 1.0, and 1.4 mmol L^{-1} NO_3^-) at 25°C over a 30-day period using methods adapted from schmidt et al. (2011).These nitrate concentrations reflect typical fluxes on a tropical reef from on-land flooding and agricultural runoff (Schmidt, 2011). Local seawater a with baseline NO_3 content of 0.5 mM will be filtered and tested for nitrate content using a Nitrate-Nitrogen Portable Photometer (Hanna Instruments model HI96728, Woonsocket Rhode Island) and adjusted to the appropriate nitrogen levels as needed by adding nutrient stock solutions of KNO_3. Nitrate-adjusted seawater will be kept in tanks and pumped through sets of 4 beakers of 20 forams each using multi-channel peristaltic pumps (Fisher Scientific FH100M, Pittsburgh, Pennsylvania) at flow rate of 50 mL min^{-1} Schmidt et al. (2011). Specimens will be exposed to each treatment for 30 days, and visually color-scored daily at 5pm with 3 individuals sub-sampled every other day for chlorophyll assay. All specimens will be color-scored and assayed at the end of the experiment. Data will be processed as before, using one-way ANOVA tests to assess whether test coloration (as measured by absorbance or mean color score) varied significantly from the beginning to the end of the treatment, and using Tukey's honestly significant difference post-hoc test to identify which time intervals yielded significant differences in coloration.

From these tests in aggregate, we will be able to determine the severity and time scale of bleaching in *M. vertebralis* in response to the most common stressors affecting reefs.

The final stage of data analysis will be to re-assess how the visual color scoring method worked in comparison to more rigorous lab methods. If the visual scoring at the end of each experiment produced results consistent with the spectrophotometric assays of the same specimens, this visual technique can be developed into a methodology for surveying foram populations, that will expedite the use of this tool even further.

Discussion

If our hypothesis is correct, and *M. vertebralis* can be shown to predictably and visibly bleach in response to the same environmental stressors that cause coral bleaching, it could revolutionize the ongoing monitoring process needed for protection of Tonga's reef ecosystems. Such a tool could potentially give scientists a useful early warning about bleaching conditions in time to proactively address controllable secondary stressors, or direct more resources towards recovery.

This research aims to establish the validity of *M. vertebralis* as a bioindicator with great potential for citizen science. If such a tool can be created, not only could monitoring for reef bleaching be simplified to monitoring a few indicative foram populations, but these populations could be assessed with fewer resources and by individuals with only basic training on sampling and color scoring. Protocols and materials can be developed so that anyone with a visual color scale key can collect meaningful data and report it to a central website or database. These citizen

science applications can help alleviate the strain on fisheries management personnel, and contribute to the greater goal of getting Tongan civilians and foreign tourists to engage in the protection of Tongan reefs. Using foram biology as an entry point for garnering interest and engagement in reef health has already proven successful during lessons taught by Frontiers Abroad students under Dr. Sharyn Goldstein in 2017, and such combined education and data collection initiatives have dual utility for Tongan fisheries managers.

Even if the development of a bio indicator does not prove promising, this research might lay the groundwork for other useful applications of forams in reef conservation. If, for example, *M. vertebralis* is found to be less sensitive to bleaching conditions than coral, it may open the door for study of forams to as interim hosts of coral zooxanthellae, and agents for symbiont repopulation in the wake of bleaching events.

Beyond applications for reef assessment, the benefits of better understanding bleaching activity in forams are numerous. Benthic forams, including *M. vertebralis*, are important models of calcareous marine life and have been used in numerous studies of the effects of changing ocean conditions (E.g. Reymond, Uthicke & Pandolfi, 2011; Vogel & Uthicke, 2012; Uthicke & Fabricius, 2012). Better understanding the myriad threats that calcifying organisms face in the context of climate change is of great importance because of their role in the carbonate equilibrium that drives the absorption and release of atmospheric carbon.

Regardless of their relevance to reef monitoring, forams like *M. vertebralis* are a prominent member of reef ecosystems, and are also threatened by climate change, with some models predicting their extinction by the end of the century (Uthicke & Fabricius, 2012). Although their greater ecological importance is not as well understood as coral, the loss of such a

prominent member of the ecosystem will surely have unforeseen consequences, and understanding their response to a shifting climate is critical to their preservation.

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